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**Impaired microvascular endothelial function is restored by acute lower-limb exercise in post-surgical
varicose vein patients**

Markos Klonizakis^a, Garry Tew^b, Jonathan Michaels^a, John Saxton^b

^aThe Sheffield Vascular Institute, Northern General Hospital, Herries Road, Sheffield, S5 7AU, U.K.

^bThe Centre for Sport and Exercise Science, Sheffield Hallam University, Collegiate Crescent Campus,
Sheffield, S10 2BP, U.K.

Short Title: Acute exercise and skin microvascular function

Contact Information:

Dr John Saxton, PhD

Centre for Sport and Exercise Sciences,

Sheffield Hallam University,

Collegiate Crescent Campus,

Sheffield, United Kingdom, S10 2BP

Fax +44 (0114) 225 4341

Phone +44 (0114) 225 4414

E-mail j.m.saxton@shu.ac.uk

Abstract

Evidence exists that cutaneous microvascular endothelial dysfunction persists in patients following varicose vein surgery. This study compared cutaneous microvascular function between post-surgical varicose vein patients and healthy controls and investigated whether any impairment of function can be attenuated by acute lower-limb exercise. Cutaneous flux responses of the gaiter area were measured in supine and standing positions before and after a 25-min walk using laser Doppler fluximetry and incremental-dose administration of acetylcholine (ACh) and sodium nitroprusside (SNP). The pre-exercise peak responses to ACh (standing) were lower in patients than controls (48 ± 11 vs. 96 ± 28 PU; $P = 0.032$), whereas treadmill exercise abolished this difference ($P = 0.819$). In contrast, the pre-exercise responses to SNP (standing) appeared higher in patients than controls (3 mC responses: 24 ± 4 vs. 10 ± 2 PU, respectively; $P = 0.023$), with no effect of acute exercise ($P > 0.05$). These findings suggest that acute treadmill exercise augments microvascular endothelial function in post-surgical varicose vein patients to levels observed in age-matched controls.

Key words: Microcirculation; Skin; Exercise; Venous disease; Laser Doppler; Endothelial function

Introduction

Chronic venous disease with skin changes of the leg is a common condition affecting up to 1 in 20 people in Western societies (Smith, 2006). Dilatation and incompetence of the deep, superficial, or perforating veins leads to impairment of the venous muscle pumps in the lower limb, often resulting in venous hypertension with upright posture (Naoum et al., 2007). Venous hypertension can activate neutrophils and monocytes, which can cause injury to the endothelium of lower-limb microvessels (Smith, 2006). Chronic injury to the endothelium can lead to a chronic inflammatory condition of the skin known clinically as lipodermatosclerosis, and skin in this state also has the potential to ulcerate in response to minor injury (Smith, 2006).

Patients with chronic venous disease often benefit from varicose vein surgery, e.g. sapheno-femoral ligation and stripping (Campbell, 2006). Indeed, venous surgery typically results in haemodynamic benefit for the legs (Gohel et al., 2005), facilitated ulcer healing (Obermayer et al., 2008), and reduced risk of ulcer

recurrence (Gohel et al., 2007). Despite these benefits, the recurrence rates for varicose veins (20.7% over an 11 yr period; Winterborn et al., 2004) and venous ulcers (18% over a 5 yr period; Nelzen and Fransson, 2007) remain relatively high. The latter might be explained, at least in part, by microvascular endothelial dysfunction that persists following surgery (Klonizakis et al., 2003; Klonizakis et al., 2006). Therefore, it appears important to identify strategies to improve microvascular endothelial function in this population.

At present, cutaneous microvascular function following varicose vein surgery is poorly characterised and there are no data regarding the effects of acute exercise on microvascular endothelial-dependent and -independent vasodilator function. Therefore, the purpose of this study was to compare cutaneous microvascular vasodilator function between patients who have recently had varicose vein surgery and age-matched controls. A secondary aim was to investigate whether any impairment of function can be alleviated by acute lower-limb exercise. In view of the existing literature (Klonizakis et al., 2003; Klonizakis et al., 2006), we hypothesised that, before acute exercise, microvascular endothelial function would be relatively depressed in patients compared to controls. Secondly, that any baseline impairment of microvascular endothelial vasodilator function would be attenuated by acute lower-limb exercise.

Methods

Participants

Twenty four patients who had recently (within 4-5 weeks) had varicose vein surgery (unilateral sapheno-femoral ligation and partial stripping) were recruited from the Sheffield Vascular Institute at the Northern General Hospital, Sheffield, UK. Twelve healthy age- and body weight-matched participants were also recruited as controls. Participants with present or past venous ulceration, lower-limb arterial disease, diabetes, hypertension, hypercholesterolaemia, peripheral oedema or cardiac failure, and those with major skin changes in the gaiter area were excluded. Participant characteristics for both groups are shown in Table 1.

This research was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and was approved by the North Sheffield Research Ethics Committee. Participants provided written informed consent according to Sheffield Hallam University guidelines.

Procedure

All assessments were performed in a temperature controlled room (range 22-24°C) following an acclimatisation period ≥ 15 min. With the participant lying supine, the gaiter area of the leg to be studied (the leg that had been operated on for the patients or the left leg for the control group) was cleaned with an alcohol wipe and allowed to dry before applying two drug delivery electrodes (PF383; Perimed AB, Järfälla, Sweden) to the surface of the leg 4-8 cm proximal to the medial malleolus. The drug delivery electrodes were positioned over healthy looking skin, approximately 4 cm apart with one containing 80 μ l of acetylcholine (ACh; Miochol-E, Novartis, Stein, Switzerland) and the other 80 μ l of sodium nitroprusside (SNP; Fagron, Hoogeveenweg, The Netherlands). Drug concentrations of 10 g·l⁻¹ were used with deionised water as the solvent. These drugs are commonly used in microvascular studies to assess endothelium-dependent and -independent vasodilator function, respectively (Turner et al., 2008). To obtain an index of skin blood flow, cutaneous red cell flux was measured by placing an iontophoresis laser-Doppler probe (PF481-1; Perimed AB), connected to a laser Doppler fluxmeter (PF5001; Perimed AB), in the centre of each drug delivery electrode. The laser-Doppler probe signals were continuously monitored via an online software chart recorder (PSW; Perimed AB).

A battery-powered iontophoresis controller (PeriIont PF382b; Perimed AB) was used to provide the charge needed for ACh and SNP delivery. Following a 4 min stable recording of baseline flux, dose-response curves for ACh- and SNP-induced vasodilation were characterised using the following protocol: 0.2 mA for 10 s (i.e., 2 mC), 0.2 mA for 15 s (i.e., 3 mC), 0.2 mA for 20 s (i.e., 4 mC), and 0.3 mA for 20 s (i.e., 6 mC), with a 4 min recording period between each dose (Fig. 1). The protocol was chosen as it is sufficient to provide effective ACh and SNP delivery but avoids the non-specific vasodilation observed with higher electrical charges (Droog et al., 2004). This protocol was then repeated with the participant in a standing position to assess the effect of posture on cutaneous microvascular function. Participants then completed a 25 min self-paced treadmill walk (Patients: 4.9 ± 0.1 km·hr⁻¹; Controls: 5.0 ± 0.1 km·hr⁻¹; $P = 0.619$) on a 0% gradient before repeating the iontophoresis protocols in both supine and standing positions to assess the effects of acute exercise on cutaneous microvascular sensitivity to ACh and SNP in different postures. The probes were moved to fresh skin areas for each body position. The peak cutaneous flux responses to ACh and SNP, measured in conventional perfusion units (PU), were used as measures of microvascular endothelial-

dependent and -independent function, respectively. The technical error of measurement for drug-induced peak flux responses in our laboratory is 15%.

Data analysis

Data are presented as mean \pm S.E.M. unless otherwise stated. Dependent variables were first tested for normal distribution using the Kolmogorov-Smirnov goodness of fit test. The majority of outcome measures were non-normal and these were log-transformed before further analysis. Differences in characteristics between patients and controls were assessed using independent *t*-tests and χ -squared tests. Group differences in cutaneous flux responses before acute exercise were assessed using mixed-model (group by dose) ANOVAs for each body position and drug, with independent *t*-tests used to interpret significant interaction effects. The acute effects of exercise on peak cutaneous flux responses in each group were assessed using mixed-model (group by time) ANOVAs for each position and drug, with paired-samples *t*-tests used to interpret significant interaction effects. Effect sizes (Cohen's *d*) were calculated for exercise-induced changes in peak cutaneous flux responses, with 0.2, 0.5, and 0.8 representing small, medium, and large effects, respectively (Mullineaux et al., 2001). Statistical significance was set at $P \leq 0.05$.

Results

Group differences in cutaneous flux responses before exercise

Resting (unstimulated) cutaneous flux in the gaiter area, both in the supine and standing positions, was not different between patients and controls. In the supine position there no differences between patients and controls for the cutaneous flux responses to ACh or SNP (data not shown; $P > 0.05$). In the standing position the peak cutaneous flux response to ACh was significantly higher in controls compared to patients (96 ± 20 vs. 48 ± 12 PU; $P = 0.032$; Fig. 2 upper panel), indicating a relatively depressed endothelial-dependent function in post-surgical varicose vein patients. In contrast, the cutaneous flux responses to SNP were generally higher in patients than controls, and this reached statistical significance for the 3 mC dose (24

± 4 vs. 10 ± 2 PU; $P = 0.023$; Fig. 2 lower panel), indicating a relatively enhanced endothelial-independent function in post-surgical varicose vein patients.

Effects of acute treadmill exercise on peak cutaneous flux responses in each group

Peak cutaneous flux responses to ACh were increased following exercise in patients, in both the supine ($P = 0.003$; Cohen's $d = 0.56$) and standing ($P = 0.004$; Cohen's $d = 0.83$) positions (Fig. 3 upper panel). In contrast, no changes were observed in the control group (Fig. 3 lower panel). After acute lower-limb exercise, the difference in peak cutaneous flux response to ACh between patients and controls in the standing position was abolished ($P = 0.819$). There were no effects of acute exercise on peak cutaneous responses to SNP in either group or body position (data not shown; $P > 0.05$).

Discussion

The present study compared cutaneous microvascular function at rest, and following acute treadmill exercise, between patients who have recently had varicose vein surgery and age-matched controls. The principle findings from this study are as follows: (i) The pre-exercise (resting) peak cutaneous flux responses to the endothelium-dependent vasodilator ACh, in the standing position, were lower in patients than in controls; (ii) the pre-exercise cutaneous flux responses to the endothelium-independent vasodilator SNP, in the standing position, were generally higher in patients than in controls; (iii) acute treadmill exercise increased microvascular endothelial function in patients in both the supine and standing positions; and, (iv) acute treadmill exercise had no effect on microvascular endothelium-independent function in either group.

In the present study, we used laser Doppler fluximetry combined with iontophoretic delivery of ACh and SNP to assess cutaneous endothelial-dependent and -independent microvascular function in the gaiter area, respectively. It is generally accepted that ACh produces cutaneous microvascular endothelium-dependent vasodilation through nitric oxide (NO)-dependent, prostanoid-dependent, and non-NO-, non-prostanoid-dependent pathways (Black et al., 2008; Cracowski et al., 2006; Holowatz et al., 2005), although the precise mechanisms remain unclear. In contrast, SNP is an NO donor that reacts with tissue sulfhydryl groups under physiologic conditions to produce NO directly and thereby stimulate smooth muscle cell

relaxation independently of the endothelium (Turner et al., 2008). The main limitation with iontophoretic drug delivery is that non-specific microvascular responses, associated with drug concentration and charge and vehicle characteristics, are common with most protocols used (Droog et al., 2004). Although we cannot exclude the possibility that our protocol elicited non-specific vasodilatory effects, the use of 10 g·l⁻¹ drug concentrations and currents ≤0.3 mA should have at least minimised such effects (Droog et al., 2004). In addition, as the protocol was consistent between groups before and after acute lower-limb exercise, it is unlikely that any observed differences/changes in microvascular responses are due to non-specific, protocol-related vasodilatory effects.

Group differences in cutaneous flux responses before exercise

The peak endothelium-dependent responses to ACh in the standing position were lower in patients than in controls (Fig. 2 upper panel). This finding is consistent with previous reports that microvascular endothelial function in the standing position is relatively depressed in patients with isolated superficial venous insufficiency compared to healthy age-matched controls (Klonizakis et al., 2003), and that venous surgery has no effect on microvascular endothelial function (Klonizakis et al., 2006). Vascular integrity in the healthy endothelium is maintained through the release of a variety of paracrine factors, such as NO. In venous disease, venous stasis in the microcirculation reduces the shear rate on the endothelial cells resulting in a reduction in cellular levels of NO (Naoum et al., 2007). This favours leucocyte adhesion and neutrophil and monocyte activation, which results in endothelial injury and dysfunction in lower-limb microvessels (Smith, 2006). In post-surgical varicose vein patients, there are high recurrence rates for varicose veins (Winterborn et al., 2004) and venous ulcers (Nelzen and Fransson, 2007). The latter might be partly explained by microvascular endothelial dysfunction (Golledge and Quigley, 2003; Smith, 2006). Therefore, it appears important to develop strategies to improve microvascular endothelial function in this population.

In contrast, the endothelium-independent responses to SNP in the standing position were generally higher in patients than controls, and significantly so for the 3 mC dose (Fig. 2 lower panel). These findings are consistent with previous reports that microvascular endothelial-independent function in the standing position is similar between varicose vein patients and healthy age-matched controls (Klonizakis et al., 2003), and that varicose vein surgery enhances cutaneous responsiveness to SNP (Klonizakis et al., 2006). The higher SNP responses of patients in the standing position are consistent with a reduction in the venoarteriolar

reflex following surgery, i.e., less activation of local neurovascular pathways that cause vascular smooth muscle contraction and oppose the effects of SNP. A reduction in the venoarteriolar reflex might be explained by differences in venous pressure as a result of surgery, or by changes in the sensory or effector arms of the reflex pathway, including down-regulation of cutaneous vasoconstrictor mechanisms (Klonizakis et al., 2006). Extracellular matrix remodelling is one of several mechanisms that might affect the balance of vasodilator and vasoconstrictor responses of the blood vessel wall (Kowalewski et al., 2004). Alternatively, the lower SNP responsiveness in controls might be explained by a relative nitrate tolerance compared to patients (Laursen et al., 1996).

Effects of acute treadmill exercise on peak cutaneous flux responses

Following a 25-min moderate-intensity treadmill walk, the peak cutaneous flux responses to ACh were increased in patients in both the supine and standing positions (Fig. 3 upper panel). This is the first study to report an improvement in microvascular endothelial function following acute lower-limb exercise in patients who have recently undergone surgical varicose vein treatment. Furthermore, the moderate-to-large effect sizes that were observed in the supine (Cohen's $d = 0.56$) and standing positions (Cohen's $d = 0.83$) suggests that these changes could potentially be clinically meaningful with respect to varicose vein recurrence and/or venous ulceration. No such changes were observed in the control group (Fig. 3 lower panel), which is consistent with some (Colberg et al., 2006; Rossi et al., 2002), but not all (Kvernmo et al., 1998), previous studies. As a result, the impairment of microvascular endothelial vasodilator function that was observed in the patients before exercise was abolished by acute lower-limb exercise, such that there was no difference in the peak perfusion responses to ACh between patients and controls. Finally, there was no effect of acute exercise on SNP responsiveness in either group or position, which is consistent with the literature (Kvernmo et al., 1998; Rossi et al., 2002). These findings suggest that moderate-intensity lower-limb aerobic exercise is an effective stimulus for improving microvascular endothelial function in post-surgical varicose vein patients; however, further research is needed to clarify the clinical relevance of these findings, i.e., whether chronic exercise training reduces recurrence rates for varicose veins and/or venous ulcers.

Various mechanisms might explain the increased microvascular endothelial function following acute exercise in the patient group. Firstly, during exercise, increased microcirculatory flow and the corresponding

increase in shear stress to the vessel walls are stimuli that elicit endothelial-dependent vasodilation (Green et al., 2004). Mechanical alteration/deformation of the endothelium during exercise as a result of increased pulsatile flow could also contribute to increased release of endothelium-derived hyperpolarizing factor (EDHF) and endothelial NO synthase upregulation (Green et al., 2004). Therefore, increased cutaneous blood flow during exercise might alter endothelial function through increased EDHF and NO bioavailability. Another potential mechanism is reduced sympathetic nervous system (SNS) activity. Neurogenic sympathetic regulation is one of the main mechanisms responsible for maintenance of peripheral vessel tone (Krupatkin, 2006). Given that SNS activity is decreased for several hours following a bout of exercise (Pober et al., 2004), it is possible that the observed improvements in microvascular endothelial vasodilator function are partly explained by attenuation of sympathetic outflow. Further research is needed to clarify the exact role of each of these potential mechanisms.

Limitations

Although we questioned participants about their health status, we did not take any measures of body fatness, blood pressure, or blood cholesterol status. Therefore, it is possible that these confounding influences on microvascular function might have impacted upon the results of this study. Another limitation was that we did not measure blood pressure or maximal perfusion responses during the microvascular function test. This prevented us from presenting data as cutaneous vascular conductance (CVC) normalised to maximal perfusion, which Cracowski et al. (Cracowski et al., 2006) believe is the optimal method of data presentation in laser Doppler studies. However, as autoregulatory processes (e.g., the myogenic response) exist to maintain a relatively constant microcirculatory flow (Schubert and Mulvany, 1999), changes in brachial artery blood pressure are unlikely to be representative of changes in microcirculatory blood pressure. We did not measure maximal perfusion because this is usually achieved through sub-dermal infusion of SNP and/or by local heating at 42-44 °C for 30 min (Cracowski et al., 2006), neither of which were appropriate to our acute exercise experimental model. Nevertheless, we believe that this does not detract from our findings in any way. Another potential limitation is that the relative intensity of exercise for each group was unknown. Therefore, the contrasting effects of acute exercise on microvascular responsiveness might be due to group differences in the relative intensity of exercise. However, this is unlikely given that absolute walking speeds were similar and that participants were well-matched for age, body mass, stature, and activity status. Finally,

further research with post-surgical varicose vein patients would be needed to clarify the duration for which microvascular endothelial function is enhanced following acute exercise, and the effects of exercise characteristics (i.e., mode, intensity, duration) on microvascular function.

In summary, we observed an impairment of microvascular endothelial function in the gaiter area of post-surgical varicose vein patients in the standing position which was abolished after a 25-min moderate-intensity treadmill walk. Although, the mechanisms of improved microvascular endothelial function following acute exercise are unclear, they are likely to be associated with enhanced NO bioavailability and/or attenuation of SNS outflow. Further research on the acute and chronic effects of exercise for post-surgical varicose vein patients is warranted to establish the clinical relevance of these findings.

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